POLY(A)$^+$ RNA FROM SHEEP OMASAL EPITHELIUM INDUCES EXPRESSION OF A PEPTIDE TRANSPORT PROTEIN(S) IN *XENOPUS LAEVIS* OOCYTES

by

YuanXiang Pan

Thesis submitted to the Graduate Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Animal Science

APPROVED:

E. A. Wong, Co-Chairman	K. E. Webb, Jr., Co-Chairman
J. R. Bloomquist	J. H. Herbein

August, 1996
Blacksburg, VA

Key Words: Sheep, Omasum, Peptide, Transport, Electrophysiology

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(ABSTRACT)

In order to verify the research from this laboratory that sheep omasal epithelium contains mRNA encoding for a peptide transporter (s) and to determine di- to octapeptide transport capability, poly(A)$^+$ RNA isolated from sheep omasal epithelium was injected into *Xenopus laevis* oocytes. Poly(A)$^+$ RNA was functionally expressed in *Xenopus* oocytes 4 to 7 d post-injection. Peptide (5 di-, 10 tri-, 6 tetra-, 2 penta-, 1 hepta-, 1 septa-, 1 octapeptide) transport capability was measured by impaling oocytes with a microelectrode to monitor membrane potential (V_m). Oocytes were maintained in pH 5.5 buffer. Peptide transport was identified as being expressed when, in the presence of a buffered peptide substrate (1 m*M*), the oocyte membrane showed persistent depolarization (a more positive V_m). In the absence of peptide transport, the membrane became depolarized with the addition of buffered substrate, but rapidly repolarized to the resting potential. Peptide transport was expressed for some di-, tri-, and tetrapeptides.

Measured depolarization ranged from 9.6 mV to 42.1 mV. Larger peptides were not transported by the oocytes. When transport expression was measured with the substrates in a pH 7.5 buffer, no transport occurred indicating that transport was dependent on a proton gradient. The data indicate that sheep omasal epithelium contains mRNA that code for a protein(s) capable of proton-dependent di-, tri-, and tetrapeptide transport. This provides further evidence that absorption of peptides from the ruminant stomach is possible.

Key Words: Sheep, Omasum, Peptide, Transport, Electrophysiology

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Drs. E. A. Wong and K. E. Webb, Jr., my committee chairmen, for their patience, experienced guidance, inspiration and assistance throughout my graduate training and in the preparation of this manuscript. Without their efforts and support, I would never have begun, nor continued my academic development at Tech.

I am indebted to Dr. J R. Bloomquist for providing me with the opportunity to study electrophysiology in his lab. His guidance, assistance, comments and discussions of my data allowed me to accomplish my project successfully.

I am grateful to Dr. J. H. Herbein for his interest, encouragement, and willingness to serve as my committee member.

I am grateful to Dr. J. C. Matthews for his assistance, friendship, and many valuable discussions which facilitated my research at Tech.

I especially thank Donald Shaw, Kristin Lee, Kiyoto Kurima , Martha McCollum, Sharon Bowers, and Vajira Jayawardena for their friendship, technical discussions and support during my research.

I wish to express my deepest appreciation to my wife, Hong Chen, for her support and many sacrifices which made my graduate program possible.

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INTRODUCTION

In recent years, the utilization of peptides as a source of amino acids and nitrogen has been recognized to be an important biological process in living species from micro-organisms to animals and plants (Payne and Smith, 1994). Much information has been obtained regarding the ability of the gastrointestinal tract and tissues such as liver (Lochs et al., 1986), kidney (Loch et al., 1988), and skeletal muscle (Roth et al., 1988) to use peptides. Overall, little is known about the magnitude of peptide absorption and the metabolic significance of this phenomenon in ruminants. Results from a recent study indicated that peptides constitute about 65 to 78% of the blood plasma amino acid pool in ruminants, and that the forestomach may be the primary site of peptide absorption in ruminants (DiRienzo and Webb, 1995). Carnosine and methionylglycine were shown to be transferred intact across both ruminal and omasal epithelial tissue without hydrolysis when these tissues were mounted in parabiotic chambers (Matthews and Webb, 1995). However, the four-strata structure of all forestomach epithelia makes it difficult to purify and further characterize the transporter protein(s) using conventional procedures. With the success of expression cloning techniques, the identification and characterization of peptide transporter protein(s) in the ruminant forestomach becomes possible. Using expression system, poly$(A)^+$ RNA isolated from omasal epithelia were injected into

Xenopus laevis oocytes (Matthews et al., 1996). Specific poly(A)$^+$ RNA fractions induced an increased rate of glycyl-sarcosine (Gly-Sar) absorption in mRNA-injected oocytes compared with water-injected oocytes. These data indicated that mRNA encoding for a transport protein(s) that is capable of dipeptide absorption exists in sheep omasal epithelium. In order to verify the research from this laboratory that sheep omasal epithelium contains mRNA encoding for a peptide transporter and to determine transport capability of di- to octapeptide substrates, *Xenopus* oocytes injected with poly(A)$^+$ RNA from sheep omasal epithelium were used as experimental models using electrophysiological techniques.

Chapter II

REVIEW OF LITERATURE

Transporters are a diverse group of membrane proteins that facilitate the movement of organic and inorganic solutes through the lipid bilayer of biological membranes. In both prokaryotes and eukaryotes, transport systems for organic solutes, such as sugars, amino acids, peptides, neurotransmitters and drugs, can be classified as passive or active transporters. Passive transporters include facilitated transporters and channels, which allow the diffusion of solutes across membranes. Active transporters couple solute transport to the input of energy and can be divided into two classes: ATP-dependent (or primary)and ion-coupled (or secondary) transporters. In general, ATP-dependent transporters are directly energized by the hydrolysis of ATP and translocate a heterogeneous set of substrates. Ion-coupled transporters link uphill solute transport to downhill electrochemical ion gradients and are coupled to the cotransport of H^+, Na^+, Cl^- and to the countertransport of K^+. The electrochemical ion gradients are maintained by H^+- or Na^+/K^+-ATPases located in cell membranes (Christensen, 1989).

In the past decade, the understanding of the biochemistry and molecular biology of transporters from *E. coli* has progressed significantly. The recent development of methods to detect the activity of recombinant proteins in individual cells has led to the cloning of several novel mammalian transporter cDNAs. The sequence information and the functional data from transporters have revealed similar mechanisms and common

evolutionary origins. The following review describes the known peptide transport systems in bacteria and mammals, and one of the most useful expression cloning systems in conjunction with electrophysiological measurements.

Mechanisms for Peptide Transport

Peptide Transport by Bacteria. Bacteria can be classified into two categories: Gram-negative and Gram-positive. The major permeability barrier in bacteria is the cytoplasmic membrane. Gram-negative bacteria, such as *Escherichia. coli* and *Salmonella. Typhimurium*, also protect themselves by an additional structure called the outer membrane. The outer leaflet of the outer membrane is composed of an unusual lipid, a lipopolysaccharide, rather than the usual glycerophospholipid found in most biological membranes. This asymmetric bilayer provides an efficient barrier against rapid penetration by lipophilic agents such as antibiotics (Nikaido, 1994a). The periplasmic space between the two membranes contains proteins released by the cell, such as receptors, which trap solutes. Porins, special proteins form non-specific aqueous diffusion channels across the outer membrane that permit capture of nutrients from the environment in the periplasm (Nikaido, 1994b). Peptides can serve as the sole source of both carbon and nitrogen in bacteria. Microbial transport systems involved in uptake of exogenous peptides are located in the cytoplasmic membrane (Ames et al., 1990).

Most understanding of ATP-dependent transport systems comes largely from studies of Gram-negative bacteria, especially *E. coli* and *S. typhimurium.* There are three

genetically distinct peptide transport systems, the dipeptide, tripeptide, and oligopeptide transporters. The oligopeptide transporter is the major peptide transport system. It will handle peptides with two up to five or six amino acid residues, but has no affinity for free amino acids (Hiles et al., 1987). This system is also responsible for the recycling of cell walls during growth (Goodell et al., 1987). The dipeptide transporter is relatively specific for dipeptides, and has a high affinity for dipeptides with hydrophobic amino acid residues such as leucine, methionine, and valine. It may also transport some tripeptides. The tripeptide transporter can transport di- and tripeptides and prefers tripeptides with hydrophobic amino acids (Hiles et al., 1987). In Gram-positive bacteria, such as *Lactococcus lactis*, an ATP-driven oligopeptide transport system that is capable of transporting peptides of four and up to at least eight amino acid residues was also identified (Kunji et al., 1993; Tynkkynen et al., 1993). It was found that oligopeptide transport is essential for the accumulation of all β-casein-derived amino acids in *L. lactis* (Kunji et al., 1995).

Active transport systems in bacteria can be classified on the basis of their sensitivity to osmotic shock (Ames, 1986). The peptide transport systems belong to a family of osmotic shock-sensitive (or binding protein-dependent) transport systems that each require several protein components and are probably energized by the hydrolysis of ATP. These multi-component transport systems are members of a large family, the ABC transporter or traffic ATPase superfamily (Tam and Saier, 1993). Each system requires a

specific substrate-binding protein, which is located in the periplasm and acts as the primary receptor for transport. In addition, each system normally requires one or two hydrophobic integral membrane proteins, which facilitate transport of the substrate across the cytoplasmic membrane, and one or two hydrophilic proteins associated with the cytoplasmic face of the inner membrane (Gallagher et al., 1989). The hydrophilic proteins are believed to play a role in coupling energy to the transport process. One *in vivo* study indicates that approximately two molecules of hydrolyzed ATP are consumed for every molecule of substrate transported (Mimmack et al., 1989).

A functional model for peptide transport in bacteria has been proposed (Ames et al., 1990). It is suggested that the peptide substrate crosses the outer membrane through specific or non-specific pores and reversibly binds to the binding protein in the periplasm. The binding protein will then change its conformation and increase its affinity for the membrane complex. This change will trigger the membrane-bound complex to form a pore and the peptide substrate will be released from the binding protein, passing through the complex pore into the cell.

Ion-coupled peptide transport was studied in Gram-positive bacteria, such as *L. lactis* and *Listeria monocytogenes*. *L. lactis* is a multiple-amino-acid auxotroph and requires an exogenous nitrogen source for growth. Most strains of *L. lactis* can use amino acids, peptides or both to satisfy this requirement. The growth yield and specific growth rate of some *L. lactis* strains can be increased when peptides are supplied instead of

amino acids (Otto, 1981). *L. monocytogenes* is a psychrotrophic bacterium with regard to foods and a common food-borne pathogen of raw and minimally processed foods. Whereas most bacteria are able to synthesize all 20 amino acids necessary for protein biosynthesis by utilizing inorganic ammonium salts as a nitrogen source, *L. monocytogenes* requires, in addition to a sugar, vitamins, and iron, five amino acids for growth (Beumer et al., 1994).

Different models for the mechanism of peptide utilization in *L. lactis* have been proposed, and one of these experimental systems involves the use of membrane vesicles. Dipeptide transport was studied in *L. lactis* membrane vesicles fused with liposomes containing cytochrome c oxidase as a proton-motive-force-generating system. With this system, a 40-fold accumulation of Ala-Glu was observed in response to a proton motive force. Addition of ionophores and uncouplers resulted in a efflux of the accumulated dipeptide, indicating that Ala-Glu accumulation was directly coupled to the proton motive force. Accumulated Ala-Glu was exchangeable with externally added Ala-Glu, Glu-Glu, and Leu-Leu, while no exchange occurred on addition of the amino acid glutamate or alanine (Smid et al., 1989a). By isolating a peptide transport-deficient mutant by selection for resistance to the toxic dipeptide L-Ala-β-chloro-Ala, these researchers also found that transport of di- and tripeptides was essential for the growth of *L. lactis* when casein was the sole N source (Smid et al., 1989b). The gene encoding this lactococcal di- and tripeptide transport system was cloned and sequenced. The translated

sequence corresponds to that of a protein of 463 amino acid residues, and hydropathy profiling indicated that the protein could form 12 membrane-spanning segments typical of a secondary transport system (Hagting et al., 1994).

The amount of free amino acids essential to *L. monocytogenes* in foods, mainly dairy products, meat, and raw vegetable is inadequate (Farber and Peterkin, 1991). Therefore, the supply of essential amino acids for growth of *L. monocytogenes* in these food must originate from other sources, possibly by degradation of proteins by other microorganisms. A mixture of peptides and amino acids will become available as the consequence of breakdown of proteins and peptides, which can supply amino acids for growth. *L. monocytogenes* has been shown to express a peptide transport system which could take up di- and tripeptides via a proton motive force-dependent carrier protein, that exhibits broad specificity with regard to the nature of the amino acids that constitute the peptide. It allowed transport of the nonpeptide substrate 5-aminolevulinic acid, the toxic di- and tripeptide analogs, alanyl-β-chloroalanine and alanyl-alanyl-β-chloroalanine, respectively, in addition to other di- and tripeptides (Vereul et al., 1995). This peptide transport system resembles the secondary di- and tripeptide transport system of *L. lactis*.

Peptide Transport by Mammals. For vertebrate epithelial cells, three general routes have been proposed which described carrier-dependent transport of peptide substrates across the cell membrane (Daniel et al., 1992; Minami et al., 1992). They are (1)

transport of intact peptides is facilitated by diffusion followed by intracellular hydrolysis, (2) transport of intact peptides is by an active transport system followed by intracellular hydrolysis, and (3) peptides are hydrolyzed during their translocation across cell membranes. The first two routes will require cytosolic peptide hydrolases and the third one will require the presence of membrane-bound hydrolases (Raghunath et al., 1990).

With facilitated diffusion, transporters regulate the downhill transport of peptide substrates into the cell. This mode of transport is dependent on the substrate concentration, but not on the hydrolysis of ATP. Data from the African tilapia suggested that, in intestinal brush border membrane vesicles (BBMV), di- and tripeptides were transported intact by a cation-independent facilitated diffusion mechanism (Reshkin and Ahearn, 1991). The study of glutathione transport in rabbit intestinal BBMV demonstrated that glutathione is translocated by a Na^+-independent transporter. Glutathione transport was optimal at pH 7.5 in either inward or outward proton gradients. Monovalent cations Li^+, K^+, and Na^+ stimulated glutathione uptake less than did the divalent cations Ca^{2+} and Mn^{2+}. This suggests that glutathione transport was mediated by a carrier-dependent, energy-independent facilitative transporter (Vincenzini et al., 1989).

Although active solute transport across plasma membranes of high vertebrates has been thought to be driven primarily by the Na^+ gradient, it is increasingly recognized that

there are also H^+-coupled transporters. H^+ electrochemical gradients are formed in epithelial cells by Na^+/H^+ exchanger at the expense of the energy stored in the Na^+ electrochemical gradient.

The study of hydrolysis-resistant peptides in BBMV has shown that peptide transporters are electrogenic and require a proton motive force to drive translocation. These H^+/peptide symporters are found in guinea pig (Himukai et al., 1983), rabbit (Ganapathy et al., 1984), and rat (Said et al., 1988) intestinal BBMV. The potential for the function of intact peptide absorption to exist in the forestomach epithelium of ruminants was also found (Matthews and Webb, 1995). Size-fractionated poly$(A)^+$ RNA isolated from omasal epithelial tissue of sheep were injected into defolliculated *Xenopus laevis* oocytes and specific RNA fractions induced an increased rate of Gly-Sar absorption in mRNA-injected oocytes compared to water-injected oocytes. This result indicated that sheep omasal epithelium expressed mRNA encoding for a transport system that is capable of dipeptide absorption (Matthews et al., 1996). A peptide transport system in ovine enterocyte BBMV has been examined with Gly-Pro as a substrate. Peptide transport, as shown by Gly-Pro uptake, was saturable and stimulated by an inwardly directed H^+ gradient (Backwell et al., 1995).

Using expression cloning with *Xenopus laevis* oocytes to screen a rabbit intestinal cDNA libraries for the uptake of the biologically inert [^{14}C]-labeled dipeptide glycyl-sarcosine (Gly-Sar), cDNAs coding for a rabbit intestinal peptide transporter called

PepT1 (Boll et al., 1994; Fei et al., 1994; Liang et al., 1995), and a human kidney isoform, PepT2 (Liu et al., 1995) were isolated. Like most mammalian co-transporters, the amino acid sequences of PepT1 and PepT2 predict a membrane protein with twelve membrane-spanning domains. However, PepT1 and PepT2 protein has an unusually large hydrophilic loop with several N-glycosylation sites and its sequence does not have significant homology to other known mammalian sequences. Comparative amino acid sequence analysis has shown that H^+/ peptide transporters form a novel family called "proton-dependent oligopeptide transport" (POT) family which is distinct from other secondary transporter families (Paulsen et al., 1994).

When expressed in *Xenopus* oocytes, these peptide transporters strongly increased the uptake of [^{14}C]-labeled dipeptide Gly-Sar (100μM). Two-electrode voltage-clamp analysis of oocytes injected with rabbit (Fei et al., 1994) and human (Mackenzie et al., 1996) PepT1 cRNA demonstrated that PepT1-mediated transport is electrogenic. Large inward currents were obtained when substrates such as dipeptides, tripeptides and β-lactam antibiotics (1mM) were applied to the bath. Because transport is electrogenic, it is possible to screen a large number of compounds quickly to establish the substrate range of PepT1. Peptides were transported, regardless of whether they contained acidic, basic or hydrophobic amino acids. It seems that any di-, tri- and tetrapeptide can be a substrate. However, the affinity among peptides varied

substantially. PepT1 appears to have a preference for peptides containing bulky aliphatic side-chains (Fei et al., 1994; Mackenzie et al., 1996).

H^+ cotransport of PepT1 was directly demonstrated by measuring intracellular pH (pHi) of oocytes using a pH-sensitive microelectrode filled with a hydrogen-selective ionophore. Application of 10 mM Gly-Sar to PepT1 cRNA-injected oocytes caused pHi to decrease from 7.22 to 7.0 and the oocyte membrane to depolarize (Fei et al., 1994).

The presence of a proton-coupled electrogenic high-affinity peptide transporter in the apical membrane of kidney tubular cells was demonstrated by microperfusion studies and in studies using isolated BBMV (Daniel et al., 1992). The transporter was found to operate in an electrogenic mode by coupling of substrate influx to an inwardly directed proton gradient. Besides di- and tripeptides, a variety of peptide mimetics with a peptide backbone were found to interact with the transporter's substrate-binding site (Daniel and Adibi, 1994). Injection of poly$(A)^+$ RNA isolated from rabbit kidney cortex into *Xenopus* oocyte resulted in expression of a pH-dependent transport activity for the uptake of di- and tripeptides and amino-cephalosporin antibiotics (Boll et al., 1996). After size fractionation of poly$(A)^+$ RNA, the transport activity was found in the 3.0- to 5.0-kb fractions, which were used for construction of a cDNA library. A cDNA (rPepT2) that codes for a 729-amino acid protein with an apparent monomer molecular mass of 107 kDa and 12 membrane-spanning domains was cloned. It showed significant

homology to other members of the POT family of proton-dependent oligopeptide transporters.

There are three possibilities for the cell to translocate peptides without carriers through the carrier-independent transport system which include endocytosis, non-mediated diffusion through existing membrane-spanning channel proteins, and paracellular pathways. Non-specific endocytosis of food-type proteins was shown by a degradative lysosomal pathway and a unidirectional basal-to-apical direct pathway in Caco-2 cells. A small proportion (<10%) was transported in an intact form (Heyman et al., 1990). The entry of peptides into red blood cells was non-saturable and non-specific suggesting that uptake was a non-mediated diffusion (Lochs et al., 1990). A glucose-dependent peptide transport, the paracellular pathway, was observed in the small intestine of the hamster (Atisook and Madara, 1991). These researchers found that the hemepeptide selectively appeared in the paracellular space where the tight junctions were dilated when the intestinal epithelium was exposed to glucose.

Peptide Structure and Competition in the Transport Process

The effects of molecular structure on the uptake of peptides have been actively studied. It has been shown that blocking either the amino or carboxyl terminal group could reduce transport affinity and lipophilic side-chains could increase uptake affinity (Guyer et al., 1986). Peptides with α-amino groups have higher affinity than peptides with β-amino groups. Peptides with D-amino acid residues have much lower affinity

than peptides with corresponding L-amino acid residues. In some instances, the order of the amino acids in the peptide was important. For example, Lys-Gly was absorbed more rapidly than Gly-Lys by rat small intestine (Burston et al., 1972).

In rat renal brush border membrane vesicles, the transport rate of the tripeptide, Tyr-Pro-Phe, across membrane was six times greater than that of the dipeptide, Tyr-Pro. However, Tyr-Pro-Phe-Pro, a tetrapeptide, could not be transported across the membrane. The uptake of both Tyr-Pro and Tyr-Pro-Phe could be inhibited to almost the same extent in the presence of other di- or tripeptides. Therefore, a common transporter was probably responsible for the transport of di- and tripeptides (Tiruppathi et al., 1991).

In kidney BBMV, peptide transporters have been shown to exist in the high and low affinity systems. Several structural features of peptide substrates determine their affinity for the high-affinity type transporters (Danniel et al., 1992). The uptake of Ala-Gly, Glu-Gly, Gly-Arg, Asp-Gly, Lys-Gly with the free and charged amino group of the N-terminal amino acid in the α-position was about 100-fold greater than the uptake of corresponding peptides with the amino group in the β-position. Dipeptides containing amino acids which favor formation of the trans-peptide bond whose bonding distance is 1.32 nm were preferred substrates. The formatiom of the peptide bond with the imino-group of Pro as in Gly-Pro or the addition of a CH_3 group as in Gly-Sar reduced affinity. Hydrophobicity is another important factor in determining affinity. Peptides with more

hydrophobic side chains have a higher affinity. The lower affinity of dipeptides containing charged COOH-terminal side groups and negatively charged NH_2-terminal side chains was mainly due to their higher polarity rather than the presence of a positive or negative charge. $Gly_{(n)}$-, $Ala_{(n)}$-, and $Leu_{(n)}$-peptides were used to determine the impact of the backbone chain length on affinity. Gly-Gly-Gly had a slightly lower affinity than Gly-Gly, but di- and tripeptides of Ala and Leu had the same affinity. Tetrapeptides had much lower affinity than the corresponding di- and tripeptides.

Results from rabbit intestinal and renal brush border membrane vesicles showed that unlabeled Gly-Pro or His-Pro could reduce by 50 to 70% the transport of radio-labeled Gly-Pro. However, Leu-Gly only decreased by 20% the transport of radio-labeled Gly-Pro (Ganapathy et al., 1981). In another study, the results showed that all peptides examined, including dipeptides (Gly-Leu, Gly-His, Gly-Phe) and tripeptides (Gly-Phe-Ala, Gly-Leu-Tyr), significantly inhibited the transport of Gly-Pro across the rat intestinal brush border membrane (Said et al., 1988). Studies with renal BBMV showed that the transport of Gly-Pro by the high affinity/low capacity transporter could be inhibited by Gly-Gly, but Gly-Gly showed no effect on the transport of Gly-Pro mediated by the low affinity/high capacity transporter (Skopicki et al., 1991). Daniel et al. (1991) found that all glycine-containing dipeptides tested inhibited uptake of Gly-Gln by renal BBMV of rats and the inhibition ranged from 63 to 86%. Another study

observed a similar range of inhibition in the uptake of Gly-Gln by intestinal BBMV of human in the presence of other di- and tripeptides (Minami et al., 1992).

Expression of the cloned rabbit intestinal H+/oligopeptide cotransporter rPepT1 in *Xenopus* oocytes revealed high apparent affinity for the anionic dipeptide alanyl-aspartate (Boll et al., 1996), whereas in rabbit intestinal BBMV neutral dipeptides, or those bearing a single positive charge, were generally favored (Wootton and Hazelwood, 1989).

Techniques Involved in Electrophysiological Recording

The use of microelectrodes as a means of probing the physiological properties of cells has grown enormously since the pioneering working of Ling & Gerard (1949) and Hodgkin & Huxley (1952). Micro-electrodes are now used in many areas of the biological sciences, such as, 1) to determine not only the membrane properties of cells with voltage clamp techniques, but also to measure the intracellular free ion concentrations of important inorganic ions, 2) to study the overall architecture of cells by injection of markers, 3) to determine the direct connections between cells and, 4) to examine membrane properties at the level of single ion channels using the patch clamp.

A microelectrode is an electrode constructed with a tip having the dimensions of the order of a micrometer. Glass microelectrodes are usually filled with a salt solution. Data from most electrophysiological experiments are recorded as changes in voltage or current over time. With appropriate amplifiers these signals are converted into a

proportional voltage signal and such signals are visualized and stored (Standen et al., 1987).

Most progress in the understanding of ion currents through cellular membranes has been achieved by the development of voltage clamp techniques. In general, the membrane potential is held at a preselected value (holding potential). Then, ion currents are activated by sudden changes in potential or composition of the solution surrounding the cells. As long as the cellular potential is changing, the membrane current is composed of currents through ion channels as well as capacitive currents. To eliminate interference from capacitive currents, the change in membrane potential has to occur before the ion channels activate. However, the maximal speed of voltage change of the membrane is limited by the maximal current flow and the capacitance of the cell (Standen et al., 1987).

Model System for Electrophysiological Recording

Use of Xenopus Oocytes as a Model System. One of the first and still most widely used assay system for quantifying an authentic protein biosynthetic processes is the fully grown oocyte of the South African clawed frog, *Xenopus laevis.* The value of *Xenopus laevis* first became apparent in 1971 when Gurdon and co-workers discovered that the oocyte constitutes an efficient system for translating foreign messenger RNA (Gurdon et al., 1971).

The *Xenopus* oocyte is a cell specialized for the production and storage of proteins for later use during embryogenesis. In addition, the complex architecture of the

frog oocyte includes the subcellular systems involved in the export and import of proteins. Therefore, the mRNA-microinjected oocyte is an appropriate system in which to study the synthesis of specific polypeptides, as well as the storage of particular proteins in various subcellular organelles and the export of others into the extracellular space. Moreover the subcellular compartmentalization, as well as the structure and biochemical, physiological, and biological properties of the synthesized protein, may be examined in the injected oocyte.

The biochemical conversion of substrates provides sensitive ways to detect minute amounts of a newly synthesized enzyme and numerous proteins with authentic enzymatic activities have been shown to be produced in *Xenopus* oocytes from foreign mRNAs. These include cytoplasmic and lysosomal enzymes, integral membrane enzymes, and secreted enzymes.

Application of Electrophysiological Methods in Oocytes. The primary advantage of using *Xenopus* oocytes for the expression of transporters is the ability to perform detailed electrophysiological recording using an *in vivo* system. In the simplest arrangement, the membrane is penetrated with a single microelectrode and the membrane potential is measured. The oocytes can be easily penetrated with two microelectrodes. This arrangement allows the use of one of the two classical methods: current clamp or voltage clamp. Most electrophysiological studies on oocytes were performed using the two-electrode voltage clamp. The large size of the oocytes also permits extracellular recording

of currents flowing through the cell membrane at various locations using a vibrating probe. The patch clamp method has been successfully applied in devitellinized oocytes for the study of single channels (Hammill et al., 1981).

Whole-cell voltage clamping of oocytes involves using two electrodes inserted into the oocyte. The large size of the oocyte (about 1 mm in diameter and .5 to 1 μL in volume for stage V oocytes) make this feasible, and is both the major advantage and disadvantage of the system. The advantage is that it is possible to insert multiple electrodes and injection needles into the same oocyte. Therefore, modulators of channel function can be injected inside the cell while recording, so that a rapid and direct response to an intracellular signal can be observed. The disadvantage is that the large size results in an extremely large membrane capacitance (about 150-200 nF), which causes a slow clamp settling time following voltage shifts. This makes it difficult to obtain any data during the first 1 to 2 msec of a depolarization, the time during which rapidly activating voltage-sensitive channels such as the sodium channel open. The large capacitance is not a serious problem in examining slow responses or ligand-gated responses in the absence of voltage shifts (Stuhmer, et al., 1992).

The most detailed electrophysiological analysis of channel function in oocytes is to examine individual channel molecules through the use of single channel recording. For recording single channels, electrodes with a small tip diameter of about 1 μm are used to make a gigaohm seal with the oocyte membrane. Oocyte patches can be analyzed in

either the cell-attached or excised configuration, as in mammalian cells. The patches can be excised in either of two configuration, inside-out which allows access from the bath to the intracellular surface of the membrane or outside-out which allows access from the bath to the extracellular surface of the membrane. Once the patch is excised, recording single channels is comparable to recording from mammalian cell patches (Rudy and Iverson, 1991).

Competition of mRNAs in Micro-Injected Oocytes. It should be taken into consideration that the injected mRNAs compete with the oocyte mRNAs, and with other injected mRNAs, for formation of polysomes in the oocyte. This competition is more crucial when mRNAs for secretory or membrane-bound proteins are injected, since the amount of polysomes for such mRNAs is rather limited in the oocytes (Richter et al., 1983).

It is also possible that more than a single mRNA species might be required to produce the catalytically active enzyme pursued. The additional mRNA(s), which could be either an oocyte or a foreign mRNA species, might then become the limiting factor in the biosynthesis of the enzyme (Asselbergs et al., 1979). The large variety of heterologous proteins made in micro-injected oocytes, the competition seen between different injected mRNAs, and the competition between injected and endogenous mRNAs suggest that all mRNAs use at least some common machinery. However, the different competition effects observed were not equal. Thus, Laskey et al.(1977) showed that

while injecting increasing amounts of globin mRNA into oocytes resulted in more globin synthesis, endogenous protein synthesis decreased with essentially reciprocal kinetics. Therefore, it is difficult to saturate the oocytes' capacity to translate globin mRNAs, because the oocyte has no spare translational apparatus. The membrane-free protamine mRNA is similar to globin mRNA in this respect (Gedamu et al., 1978), but the capacity of oocytes to translate the membrane-associated zein mRNAs is saturated at relatively low amounts of injected mRNA (Larkins et al., 1979). Hybridization of RNA from injected oocytes to cDNA probes revealed that there was no difference in the stability of globin and zein mRNAs and that most of the globin mRNA was associated with polysomes, whereas most of the zein mRNA was not. Thus, the oocyte appears to be limited in rough endoplasmic reticulum (RER). Indeed, micro-injection of RER induced zein synthesis in the injected oocytes (Larkins et al., 1979).

Degradation of Foreign mRNAs in Micro-Injected Oocytes. When different types of radioactive RNAs were injected into oocytes and their degradation followed with time, it was found that ribosomal RNAs were degraded rapidly, with apparent first-order kinetics with 1 to 6 h half-lives. Whole ribosomal particles were rather stable for 20 h and poly(A)$^+$ RNAs displayed biphasic kinetics, with about 60% degrading rapidly and the rest were rather stable for at least 20 h (Allende et al., 1974). A minimal length of 30 adenylate residues was found to be sufficient to maintain full stability of globin mRNA chains, whereas poly(A) tails shorter than 30 did not have any stabilizing effect (Nudel et

al., 1976). In the absence of a poly(A) tail or the proteins bound to it, rapid destruction of translationally active globin mRNA takes place in the oocytes. The stability of deadenylated globin mRNA is much lower than that of native poly(A)$^+$ RNA in micro-injected HeLa cells as well (Huez et al., 1981), which indicates that this finding is not peculiarity of an oocyte, but holds true in somatic cells as well.

Another structural element that has been implicated in the stability of mRNA in the oocytes is the 5'-terminal "cap". Removing or breaking open the cap structure of globin mRNA causes a complete reduction (>95%) in its ability to direct globin synthesis in oocytes (Lockard and Lane, 1978). It has been shown that the capped species are quite stable and that cap removal greatly destabilizes all ten reovirus mRNAs. Thus, direct and indirect evidence suggests that in most cases, mRNA must be capped to be efficiently translated in oocytes (McCrae and Woodland, 1981).

In summary, the *Xenopus* oocyte system has the advantage that channels, receptors and transporters can be rapidly expressed and analyzed both biochemically and electrophysiologically in an *in vivo* situation. The system can be used quite effectively as an assay for the functional cloning of channels that have only been identified by their electrophysiological properties. Once cDNA clones have been isolated, oocytes are an excellent system for correlating structure with function using a combination of molecular biological and electrophysiological techniques.

Chapter III

POLY(A)$^+$ RNA FROM SHEEP OMASAL EPITHELIUM INDUCES EXPRESSION OF A PEPTIDE TRANSPORT PROTEIN(S) IN *XENOPUS LAEVIS* OOCYTES

ABSTRACT

In order to verify research from this laboratory indicating that sheep omasal epithelium contains mRNA encoding for a peptide transporter(s) and to determine di- to octapeptide transport capability, poly(A)$^+$ RNA isolated from sheep omasal epithelium was injected into *Xenopus laevis* oocytes. Poly(A)$^+$ RNA was functionally expressed in *Xenopus* oocytes 4 to 7 d post-injection. Peptide (5 di-, 10 tri-, 6 tetra-, 2 penta-, 1 hexa-, 1 hepta-, 1 octapeptide) transport capability was measured by impaling oocytes with a microelectrode to monitor membrane potential (V_m). Oocytes were maintained in pH 5.5 buffer. Peptide transport was identified as being expressed when, in the presence of a buffered peptide substrate (1 m*M*), the oocyte membrane showed persistent depolarization (a more positive V_m). In the absence of peptide transport, the membrane became depolarized with the addition of buffered substrate, but rapidly repolarized to the resting potential. Peptide transport was expressed for some di-, tri-, and tetrapeptides. Measured depolarization ranged from 9.6 mV to 42.1 mV. Larger peptides were not transported by the oocytes. When transport expression was measured with the substrates in a pH 7.5 buffer, no transport occurred, indicating that transport was

dependent on a proton gradient. These data indicate that sheep omasal epithelium contains mRNA that codes for a protein(s) capable of proton-dependent di-, tri-, and tetrapeptide transport. This provides further evidence that absorption of peptides from the ruminant stomach is possible.

Key Words:; Sheep, Omasum, Peptide, Transport, Electrophysiology

INTRODUCTION

It has been well documented that the omasum of ruminant animals has the ability to absorb VFA (McSweeney, 1988), ammonia and electrolytes (Oyaert and Bouckaert, 1961), and water (Holtenius and Bjornhag, 1989). However, the ability of the omasum to absorb peptides as a source of amino acid N has not been clearly defined.

Recent research has increased the understanding of the potential for peptide absorption to exist in the forestomach epithelium of ruminants. Matthews and Webb (1995) showed that carnosine and methionylglycine were transferred intact across both ruminal and omasal epithelia when these tissues were mounted in parabiotic chambers. Using expression system, poly(A)$^+$ RNA isolated from omasal epithelia were injected into *Xenopus laevis* oocytes (Matthews et al., 1996b). Specific poly(A)$^+$ RNA fractions induced an increased rate of glycyl-sarcosine (Gly-Sar) absorption in mRNA-injected oocytes compared to water-injected oocytes. The data indicated that mRNA encoding for a protein(s) that is capable of dipeptide transport exists in sheep omasal epithelia. To elucidate the transport mechanisms of a larger number of small peptides, *Xenopus* oocytes injected with poly(A)$^+$ RNA from sheep omasal epithelia were used as experimental models using electrophysiological techniques to determine di- to octapeptide transport capability.

MATERIALS AND METHODS

Total RNA Extraction from Animal Tissue. A modification of the method of Puissant and Houdebine (1990) was used to extract total RNA from the omasal epithelium of crossbred sheep (average BW 60 kg). Briefly, omasal mucosal scrapings were homogenized in 4 *M* guanidine thiocyanate, 25 m*M* sodium citrate, pH 7.0, .1 *M* β-mercaptoethanol, and .5% N-laurylsarcosinate. Homogenates were then pooled into centrifuge bottles that contained 2 *M* acetic acid (pH 4.1), water-saturated phenol, and chloroform. The suspensions were centrifuged at 12,000 x g at 4°C for 20 min. The resulting supernatants were precipitated with isopropanol at -20°C for 12 h and centrifuged for 20 min at 4,000 x g at 4°C. The pellets were resuspended in 4 *M* LiCl, and centrifuged at 3,600 x g at room temperature (~21°C) for 20 min. The RNA pellets were pooled again in 10 m*M* Tris·HCl (pH 7.6), 1 m*M* EDTA, .5% SDS, and chloroform. The solutions were then centrifuged at 12,000 x g at 4°C for 20 min. The supernatants containing total RNA were then stored at -80°C in the presence of .2 *M* sodium acetate (pH 5.0) and isopropanol. When needed, RNA was collected by centrifugation, washed in 70% ethanol, and suspended in water.

Poly(A)$^+$ RNA Isolation and Fractionation. Poly(A)$^+$ RNA was purified from total RNA on oligo(dT) cellulose following established procedures (Sambrook et al., 1989). Briefly, total RNA was recovered by centrifugation at 12,000 x g at 20°C for 25

min, and then dissolved in diethyl pyrocarbonate (DEPC)-treated water. Typically, about 10 mg of total RNA were loaded on a column which contained about 200 mg of oligo(dT) cellulose. Normally, a second round of chromatography was performed on a column which contained 80 mg of oligo(dT) cellulose to further purify the poly(A)$^+$ RNA. For both rounds, the final RNA eluate was precipitated with an equal volume of isopropanol at -80°C.

Poly(A)$^+$ RNA was fractionated on a linear sucrose density gradient (8 to 20% wt/vol) prepared according to the method of Luthe (1983). Briefly, poly(A)$^+$ RNA was recovered by centrifugation at 80,000 x g at 2°C for 30 min. A 100 µg (1µg/µ*L*) sample of poly(A)$^+$ RNA was loaded onto a 13 m*L* 8 to 20% (wt/vol) linear sucrose gradient containing 10 m*M* Tris·HCl (pH 7.6), 1 m*M* EDTA (pH 8.0), 10 m*M* methyl mercuric hydroxide and then centrifuged in a Beckman SW-41 ultracentrifuge rotor at 80,000 x g at 4°C for 15.5 h. Four fractions of 3 m*L* each were then collected. An equal volume of 5 m*M* β-mercaptoethanol was then added to each fraction. The fractionated RNA was precipitated with ethanol, 3 M sodium acetate and stored at -80°C until use.

Oocyte Preparation, Storage and Microinjection. Female *Xenopus laevis* frogs (Xenopus One, Ann Arbor, MI) were kept in water tanks at 15 to 16°C in de-chlorinated water on a 8 h light and 16 h dark cycle and fed at least three times a week with a complete diet (Frog Brittle, Nasco, Fort Atkinson, WI). Following methods described by Goldin (1992) to obtain the oocytes, mature frogs were anesthetized by immersion in ice-

water supplemented with .15% ethyl-m-aminobenzoate (MS222 from Sigma). A small incision was made in the lower abdominal quadrant and a lobule of ovarian tissue, containing up to 200 oocytes, was removed and placed in Ca^{2+}-free medium (in m*M*: 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES titrated with NaOH to pH 7.5). The wound was closed in two layers and the frog allowed to recover from the anesthesia in a separate tank containing .01% penicillin-G in water for several hours, then returned to the colony.

The ovarian material was separated into pieces and treated at room temperature (~21°C) with Collagenase A (Boerhringer Mannheim Corporation, Indianapolis, IN) at a concentration of .5 units/m*L* in Ca^{2+}-free medium for about 40 min. Oocytes at stages V and VI were collected after the surrounding tissue layers were peeled off with watch-maker's forceps (Size 5, George Tiemann and Company, Plainfield, NY). These defolliculated oocytes were stored again for at least 2 h in Ca^{2+}-free medium and the adhering follicle cells were removed by gentle shaking. After washing, oocytes were incubated (Incubator, Model BK6160, Heraeus Instruments, Inc., South Plainfied, NJ) at 18°C in a daily culture solution (in m*M*: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES titrated with NaOH to pH 7.5) supplemented with 2.5 m*M* sodium pyruvate, 100 units/m*L* penicillin-G, and 100 μg/m*L* streptomycin.

The poly$(A)^+$ RNA or size-fractionated poly$(A)^+$ RNA was dissolved in DEPC-treated water to a final concentration of 1.5 ng/n*L*. The oocytes were placed in injection buffer (in m*M*: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES titrated with NaOH to pH

7.5) in a tissue culture dish under a stereomicroscope. Using a microinjection system (Matthews et al., 1996a), 100 n*L* of either RNA solution or DEPC-treated water were injected into each oocyte in the vegetal pole, near the polar interface. The injected oocytes were returned to the daily culture solution and then incubated at 18 °C for 1 to 7 d. The culture solution was changed daily and damaged oocytes, as indicated by misshapen and ruptured oocytes, were discarded.

Electrophysiological Recording from Injected Oocytes. Conventional intracellular glass microelectrode recordings were used to monitor peptide transport via changes in the oocyte membrane potential. Potentials were monitored by an amplifier, and analyzed by a MacLab (AD Instruments, Milford, MA) which is an analog-digital converter and software system that uses an Apple Macintosh computer for performing data acquisition (Soderlund et al., 1989).

Microelectrodes were pulled from borosillicate glass filament tubing of 1.5 mm outer diameter and .87 mm inner diameter using a vertical pipette puller. The electrode was filled with 3 *M* KCl giving a tip resistance of 2 to 5 MΩ and a tip potential of no more than 5 m*V*. As long as the electrode retained its low resistance, it could be reused for several oocytes.

Normally, 4 to 7 d post injection with poly(A)$^+$ RNA or DEPC-treated water, a single oocyte was placed in a recording chamber (500 µ*L*) in the presence of 125 µ*L* pH 5.5 measurement buffer (in m*M*: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES titrated

with NaOH to pH 5.5). Oocytes were maintained in this pH 5.5 buffer for at least 20 min before impalement. Oocytes were always impaled to enable measurement of the resting membrane potential (V_m) and then allowed to stabilize before the start of the experiment. In most cases (approximately 70% of successful impalements) the electrode was inserted into the dark, animal hemisphere for better visualization of the electrode. No difference was observed between potential responses, ΔV_m from the animal or vegetal pole. Only oocytes at stage V or VI with a resting V_m more negative than -30 mV and that responded to the addition of buffer by becoming depolarized followed by rapid repolarization were used.

Screening for peptide transporters was performed by adding 125 μ*L* of appropriate 2 m*M*, pH 5.5 peptide solution to the recording chamber that contained 125 μ*L* pH 5.5 measurement buffer. Peptide solutions were prepared by dissolving the peptides in the appropriate measurement buffer. Changes in pH of the solutions resulting from dissolving peptides were compensated with NaOH or HCl. Peptide solutions flowed into the recording chamber through a plastic tube (1 mm). For treatment, the plastic tube was manually moved near the oocyte. Care was taken to minimize mechanical stimulation. To determine pH-dependency of peptide transport, the oocyte was placed either in pH 5.5 or pH 7.5 measurement buffer before impalement. Then appropriate peptide solutions were added to the recording chamber. All experiments were performed at room temperature (~21°C).

The uptake of peptide substrates (1 m*M*) from measurement buffer by oocytes was determined by the changes of oocyte V_m. Peptide transport was identified as being expressed when, in the presence of a buffered peptide substrate, the oocyte V_m showed persistent depolarization (a more positive V_m). In the absence of peptide transport, the membrane became depolarized with the addition of buffered substrate, but rapidly repolarized to the resting V_m. Similar responses were observed as early as 1982 in intestinal epithelial cells, which showed that transport of intact peptides caused a depolarization of the brush-border membrane (Body and Ward, 1982).

Peptides Examined. Twenty six peptides (di- to octapeptides), which were all constituted with L-α-amino acid residues and purchased from Sigma Chemical Co. (St. Louis, MO), were tested in this study. These peptides are listed in Table 3.1.

RESULTS AND DISCUSSION

Identification of Peptide Transport Capability in Poly(A)$^+$RNA-injected Oocytes. As an initial experiment, oocytes were injected with total poly(A)$^+$ RNA to study whether mRNA encoding for proteins capable of peptide transport could be identified by electrophysiological measurements. Among the 13 peptides examined, peptide transport was shown to be expressed for one dipeptide (Gly-Sar), a tripeptide mixture (Lys-Tyr-Lys, Gly-Leu-Tyr, Lys-Trp-Lys), and two tetrapeptides (Met-Gly-Met-Met, and Val-Gly-Asp-Glu) in oocytes injected with total poly(A)$^+$ RNA in this initial experiment (Figure 3.1). Measured depolarization ranged from 9.6 m*V* to 42.1 m*V*. Two

tetrapeptides (Val-Ala-Ala-Phe, Pro-Phe-Gly-Lys), two pentapeptides (Tyr-Gly-Gly-Phe-Met, Tyr-Gly-Gly-Phe-Leu), one hexapeptide (Lys-Arg-Gln-His-Pro-Gly), one septapeptide (Arg-Val-Tyr-Val-His-Pro-Phe), and one octapeptide (Val-His-Leu-Thr-Pro-Val-Glu-Lys) were not transported by the injected oocytes in this study. Water-injected oocytes (as opposed to poly(A)$^+$ RNA injected oocytes) did not show any persistent depolarization with any of the tested peptides, which indicated that the endogenous ability of oocytes to transport peptides was low.

Using this electrophysiological measurement, the ability to transport peptides by injected oocytes could be detected on d 4 after injection of poly(A)$^+$ RNA. Most of the injected oocytes responded to the peptide treatments on d 5 to d 7, which indicated that the expression of poly(A)$^+$ RNA in injected oocytes increased by day. In contrast, there was no clear change in V_m in water-injected oocytes by day. These data from this initial study were consistent with previous research from this laboratory (Matthews et al., 1996b). In that study, a time course trial was conducted to determine the optimal post-injection day required to measure the absorption of [^{14}C]Gly-Sar by the oocytes. Oocytes were measured for their ability to transport [^{14}C]Gly-Sar on d 1 to d 4 after injection of sheep omasal RNA or water. As compared with water-injected oocytes, the absorption of Gly-Sar by oocytes injected with RNA on d 3 and d 4 was 1.5 and 2.6 times greater, respectively.

Effects of pH Changes on Peptide Transport Capability in Poly(A)$^+$RNA-injected Oocytes. The pH dependency of the peptide transport process was investigated by testing the effects of measurement buffer and peptide solution pH on V_m during the recording. The V_m of poly(A)$^+$ RNA-injected oocyte showed persistent depolarization when peptide solutions at pH 5.5 were added to measurement buffer also at pH 5.5 (Figure 3.2a). This was observed when Gly-Sar, Lys-Tyr-Lys, Leu-Leu-Tyr, Lys-Trp-Lys, Met-Gly-Met-Met, and Val-Gly-Asp-Glu were used as the substrate. With the measurement buffer at a pH of 7.5, the membrane became depolarized with the addition of pH 7.5 peptide substrate, but rapidly repolarized to the resting potential. In water-injected oocytes, neither at pH 5.5 nor at pH 7.5, did V_m show persistent depolarization indicative of peptide uptake (Figure 3.2b).

In order to determine whether these results were due to the pH dependency of the transport process or just the responses of sequential treatments, additional experiments were conducted. One trial was performed with a completely opposite sequence, where the V_m of poly(A)$^+$ RNA-or water-injected oocyte was first recorded at pH 7.5 of measurement buffer when the peptide solution was at pH 7.5. The pH was then decreased to pH 5.5, and then a peptide solution at pH 5.5 was added (Figure 3.2c). Another trial was performed only at pH 7.5 of measurement buffer when peptide solution at pH 7.5 was added (Figure 3.2d). In both trials, only at a pH of 5.5 did V_m of poly(A)$^+$ RNA-injected oocytes show persistent depolarization. These data indicate that

increasing pH or decreasing the proton gradient reduces the peptide transport ability of poly(A)$^+$ RNA-injected oocytes. These results were consistent with previous research which indicated that the quantity of induced [^{14}C]Gly-Sar uptake by RNA-injected oocytes at pH 5.5 (91.9 +/- 32.2 fmols/oocyte·40 min^{-1}) was greater than that at pH 7.5 (-1.30 +/- 22.2 fmols/oocyte·40 min^{-1}; Matthews et al., 1996). In two other studies, the current evoked by 1 mM Gly-Sar at pH 7.5 in RNA-injected oocytes was about 30% of pH 5.5 (Mackenzie et al., 1996), and [^{14}C]Gly-Sar (30 μ*M* Gly-Sar) uptake by RNA-injected oocytes at pH 7.5 was only 54% that at pH 5.5 (Liang et al., 1995). This discrepancy may indicate that there was an appreciable H^+-uncoupled flux of Gly-Sar at pH 7.5.

Demonstration of Peptide Transport Ability in Size-fractionated RNA-injected Oocytes. The capacity of the oocytes to translate poly(A)$^+$ RNA is limited. Therefore, in order to obtain the maximal expression of sheep omasal epithelial RNA in oocytes and to enhance the recording of V_m signals, poly(A)$^+$ RNA was fractionated on a linear sucrose density gradient. This allowed more accurate sizing and better enrichment of biologically active RNA than did just poly(A)$^+$ RNA on oligo(dT) cellulose. After centrifugation, four fractions of poly(A)$^+$ RNA were collected (from fraction I to IV, the size of poly(A)$^+$ RNA was decreased) and injected into *Xenopus* oocytes. Gly-Sar, Leu-Ser-Phe, and Met-Gly-Met-Met were used to test which fraction had the ability to induce peptide transport in oocytes. Neither fraction I nor II showed any ability to elicit

a change in V_m as compared with that of water-injected oocytes (Table 3.2). Both fraction III and IV showed the ability to induce peptide transport in oocytes. Fraction III and IV in the present study included the fractions giving positive responses in earlier studies (Matthews et al., 1996b).

Dependency on Na^+ of Peptide Transport Ability in Size-fractionated RNA-injected Oocytes. The Na^+ dependency of the V_m change was investigated in size-fractionated RNA-injected oocytes. The Na^+-free solutions were prepared by replacing NaCl with choline chloride in both the measurement buffer and peptide solutions. A total of 10 peptides were used as the substrates and the change in V_m of oocytes injected with either fraction III or IV was virtually identical in Na^+-free solutions, as in Na^+-containing solutions. Examples are shown in Figure 3.3. This finding indicates that peptide transport in size-fractionated RNA-injected oocytes is independent of Na^+. There have been conflicting reports with regard to the dependence of peptide transport on Na^+. However, more and more evidence has shown that the absorption of peptides in the mammalian epithelia of intestine, kidney, lung, placenta, and the blood-brain barrier is mediated by one or more H^+-coupled transporters (Meredith and Boyd, 1995). The sequential actions of the Na^+/H^+ exchanger (Knickelbein, et al., 1983) and Na^+/K^+-ATPase generate an inward H^+ electrochemical gradient sufficient to drive the tertiary transport of peptides. The present study also showed that the inhibition of peptide transport by the total replacement of Na^+ by choline was unable to be detected by single

electrode membrane potential measurement. This finding suggests that Na^+ might not have a role in the primary translocation step of peptide transport or the single electrode membrane potential measurement is not sensitive enough to detect this difference.

Determination of Substrate Specificity in Size-fractionated RNA-injected Oocytes. In order to determine the structural features of peptides that might influence their affinity for the peptide transporter, fraction III and IV were injected into oocytes and tested with seventeen peptides. In oocytes injected with fraction III and IV, oocytes showed transport ability for the dipeptides Gly-Sar, Gly-Leu, Gly-Pro, Phe-Leu, Leu-Leu (Table 3.3). For tripeptides, oocytes showed transport ability of Leu-Ser-Phe, Leu-Gly-Phe, Lys-Tyr-Lys, Ala-Pro-Gly, and Met-Leu-Phe, Leu-Leu-Tyr. For tetrapeptides, oocytes showed transport ability for Met-Gly-Met-Met, Val-Gly-Asp-Glu, Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu, but no transport of Pro-Phe-Gly-Lys and Val-Ala-Ala-Phe.

Reports have suggested that hydrophobic peptides and peptides resistant to mucosal-hydrolysis are absorbed faster than hydrophilic and hydrolysis-susceptible peptides (Gardner and Wood, 1989; Daniel et al., 1992; Pan et al., 1996). Expression of the cloned rabbit intestinal H+/oligopeptide cotransporter rPepT1 in *Xenopus* oocyte displayed high apparent affinity for the anionic dipeptide alanyl-aspartate (Boll et al., 1996), whereas in rabbit intestinal brush border membrane vesicles, neutral dipeptides, or those bearing a single positive charge, were generally favored (Wootton and Hazelwood, 1989). In the present study, the transporter(s) had similar affinity for di- and tripeptides,

but for tetrapeptides, those with side chains containing acidic groups (such as Asp or Glu) or sulfur atoms (such as Met) are more likely to be transported. The oocytes injected with fractions III or IV had a similar transport ability for di- to tetrapeptides, which indicates that more specific fractions need to be prepared to separate transporters with different peptide affinities.

In summary, the present study has demonstrated that there are mRNA present in sheep omasal epithelial cells that encode for a peptide transporter and that peptide transport activity in RNA-injected oocytes can be detected by electrophysiological techniques. Substrate specificity of the transporter(s) indicates that many but not all di-, tri-, and tetrapeptides can be transported

Implications

The investigations of this study verify that mRNA encording for a peptide transporter(s) is present in omasal epithilium. Studying the kinetic characteristics of the peptide transporter(s) ought to provide insights into structure-function relationship for the transport protein(s). If further research verifies this peptide transport capability in animal body, then an important machanism for N supplementation may be revealed.

Table 3.1. Peptides examined and abbreviations used

Peptide[1]	Abbreviation
Dipeptide	
Glycyl-leucine	Gly-Leu
Glycyl-proline	Gly-Pro
Glycyl-sarcosine	Gly-Sar
Leucyl-leucine	Leu-Leu
Phenylalanyl-leucine	Phe-Leu
Tripeptide	
Alanyl-prolyl-glycine	Ala-Pro-Gly
glycyl-leucyl-tyrosine	Gly-Leu-Tyr
Leucyl-glycyl-phenylalanine	Leu-Gly-Phe
Leucyl-leucyl-tyrosine	Leu-Leu-Tyr
Leucyl-seryl-phenylalanine	Leu-Ser-Phe
Lysyl-tryptophyl-Lysyl	Lys-Trp-Lys
Lysyl-tyrosyl-lysine	Lys-Tyr-Lys
Methionyl-alanyl-serine	Met-Ala-Ser
Methionyl-leucyl-phenylalanine	Met-Leu-Phe
Threonyl-valyl-leucine	Thr-Val-Leu
Tetrapeptide	
Alanyl-glycyl-seryl-glutamic acid	Ala-Gly-Ser-Glu
Methionyl-glycyl-methionyl-methionine	Met-Gly-Met-Met
Prolyl-phenylalanyl-glycyl-lysine	Pro-Phe-Gly-Lys
Valyl-alanyl-alanyl-phenylalanine	Val-Ala-Ala-Phe
Valyl-glycyl-aspartyl-glutamic acid	Val-Gly-Asp-Glu
Valyl-glycyl-seryl-glutamic acid	Val-Gly-Ser-Glu
Pentapeptide	
Tyrosyl-glycyl-glycyl-phenylalanyl-methionine	Tyr-Gly-Gly-Phe-Met
Tyrosyl-glycyl-glycyl-phenylalanyl-leucine	Tyr-Gly-Gly-Phe-Leu
Hexapeptide	
Lysyl-arginyl-glutaminyl-histidyl-prolyl-glycine	Lys-Arg-Gln-His-Pro-Gly
Septapeptide	
Arginyl-valyl-tyrosyl-valyl-histidyl-prolyl-phenylalanine	Arg-Val-Tyr-Val-His-Pro-Phe
Octapeptide	
Valyl-histidyl-leucyl-threonyl-prolyl-valyl-glutamyl-lysine	Val-His-Leu-Thr-Pro-Val-Glu-Lys

[1] Constituent amino acids are of the L-form.

Table 3.2. Response of size-fractionated RNA-injected oocytes to peptides

	Peptide		
Fraction	Gly-Sar	Leu-Ser-Phe	Met-Gly-Met-Met
I[Ü]	0(6)*	0(6)	0(6)
II	0(5)	0(5)	0(5)
III	6(8)	4(8)	4(8)
IV	4(6)	3(6)	4(6)

[Ü] Fraction I is the 20% sucrose side and Fraction IV is the 8% sucrose side.

* Numerals are number of oocytes whose membrane potential was depolarized after addition of peptide. Numerals in parenthesis are number of total oocytes tested.

Table 3.3. Peptide substrate specificity in fraction III, IV-injected oocytes

	Oocyte response	
Peptide	Positive[1]	Negative[2]
Gly-Leu	7*	3
Gly-Pro	7	2
Gly-Sar	8	2
Leu-Leu	3	6
Phe-Leu	6	2
Ala-Pro-Gly	5	4
Leu-Gly-Phe	7	2
Leu-Leu-Tyr	3	5
Leu-Ser-Phe	7	2
Lys-Tyr-Lys	6	3
Met-Leu-Phe	5	4
Ala-Gly-Ser-Glu	5	3
Met-Gly-Met-Met	7	2
Pro-Phe-Gly-Lys	0	8
Val-Ala-Ala-Phe	0	7
Val-Gly-Asp-Glu	7	2
Val-Gly-Ser-Glu	6	3

[1] A positive response means the oocyte's membrane potential (V_m) showed persistent depolarization (a more positive V_m) with addition of the peptide.

[2] A negative response means the oocyte's V_m was depolarized with addition of the peptide, but then rapidly repolarized to the resting V_m..

* Numerals are number of oocytes.

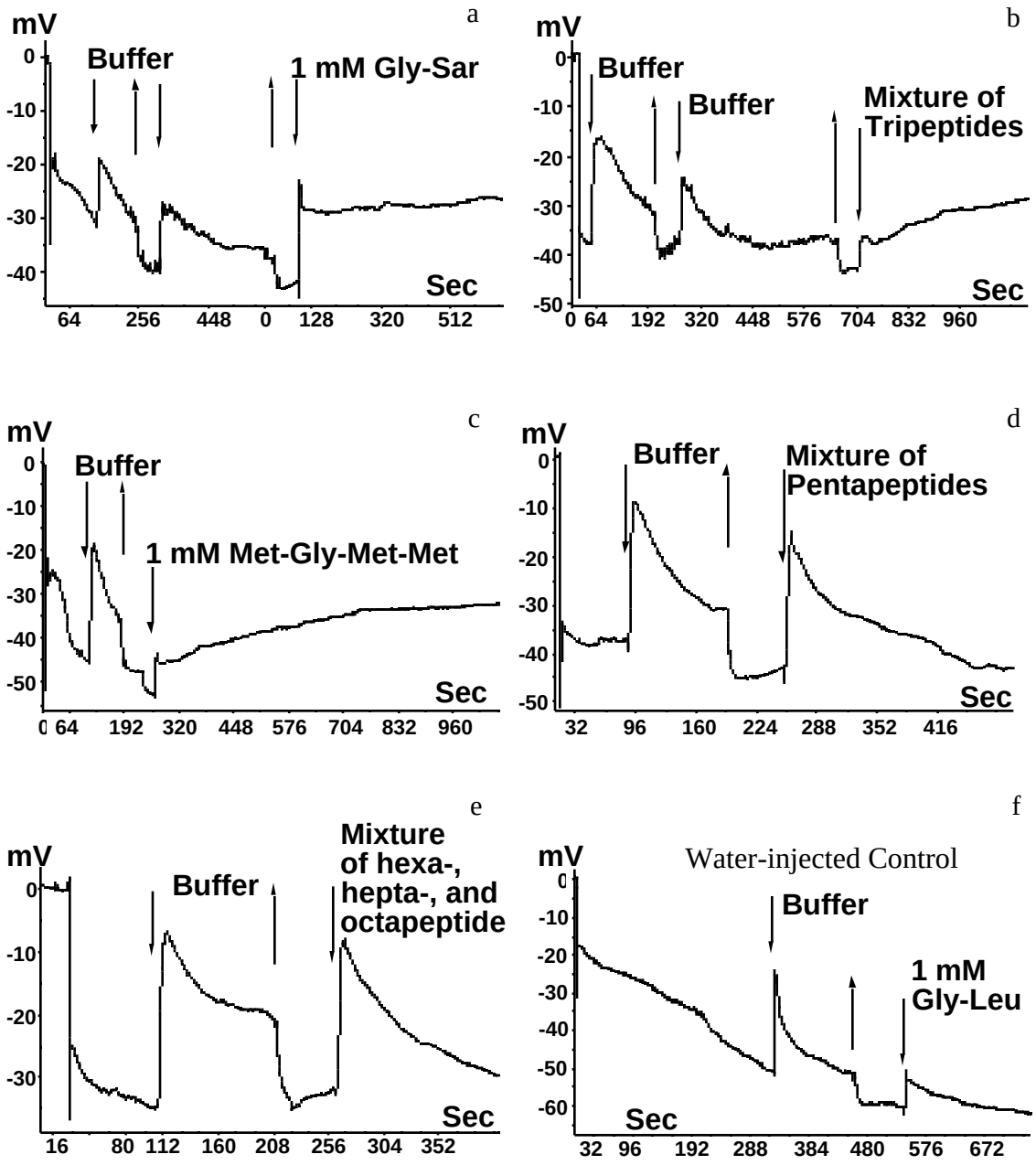

Figure 3.1. Membrane potential responses to different treatments of di-(a), tripeptide mixture(b), tetra-(c), penta-(d), mixture of hexa-, hepta-, octapeptides(e) in *Xenopus* oocytes 5 d after injection with sheep omasal epithelial total poly(A)$^+$ RNA or water(f). Oocytes were measured in pH 5.5 buffer. For all figures, downward arrows(↓) indicate time of treatment addition, upward arrows(↑) indicate time of buffer removal.

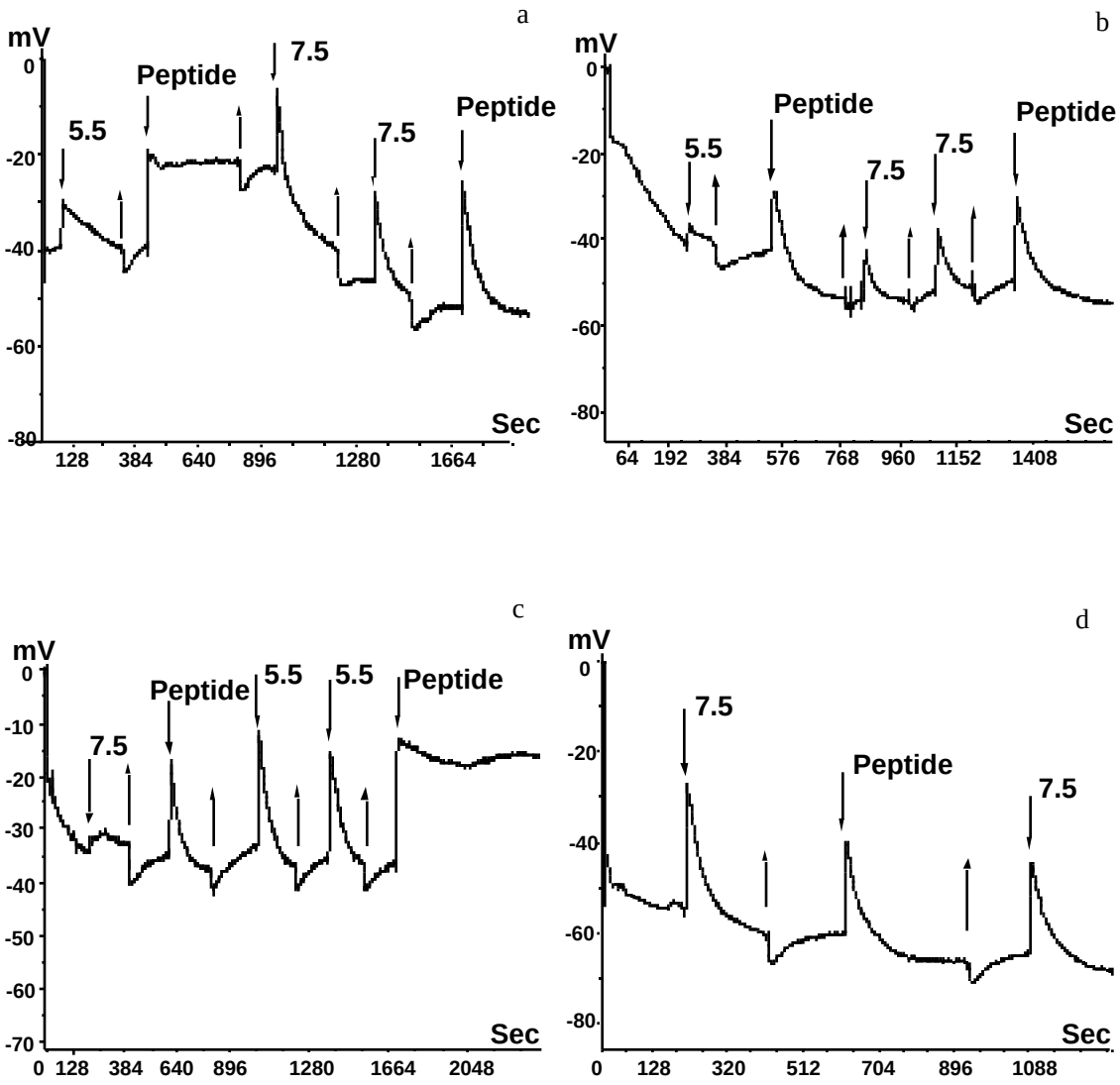

Figure 3.2. Responses to different peptide treatments at various proton gradients in *Xenopus* oocytes 5 to 7 d after injection with sheep omasal epithelial poly(A)$^+$ RNA or water. The change of pH from 5.5 to 7.5 in poly(A)$^+$ RNA-injected oocytes(a) or water-injected oocytes(b) and pH change from 7.5 to 5.5(c) or pH at 7.5 only(d) in poly(A)$^+$ RNA-injected oocytes are shown. For all figures, downward arrows(↓) indicate time of treatment addition, upward arrows(↑) indicate time of buffer removal.

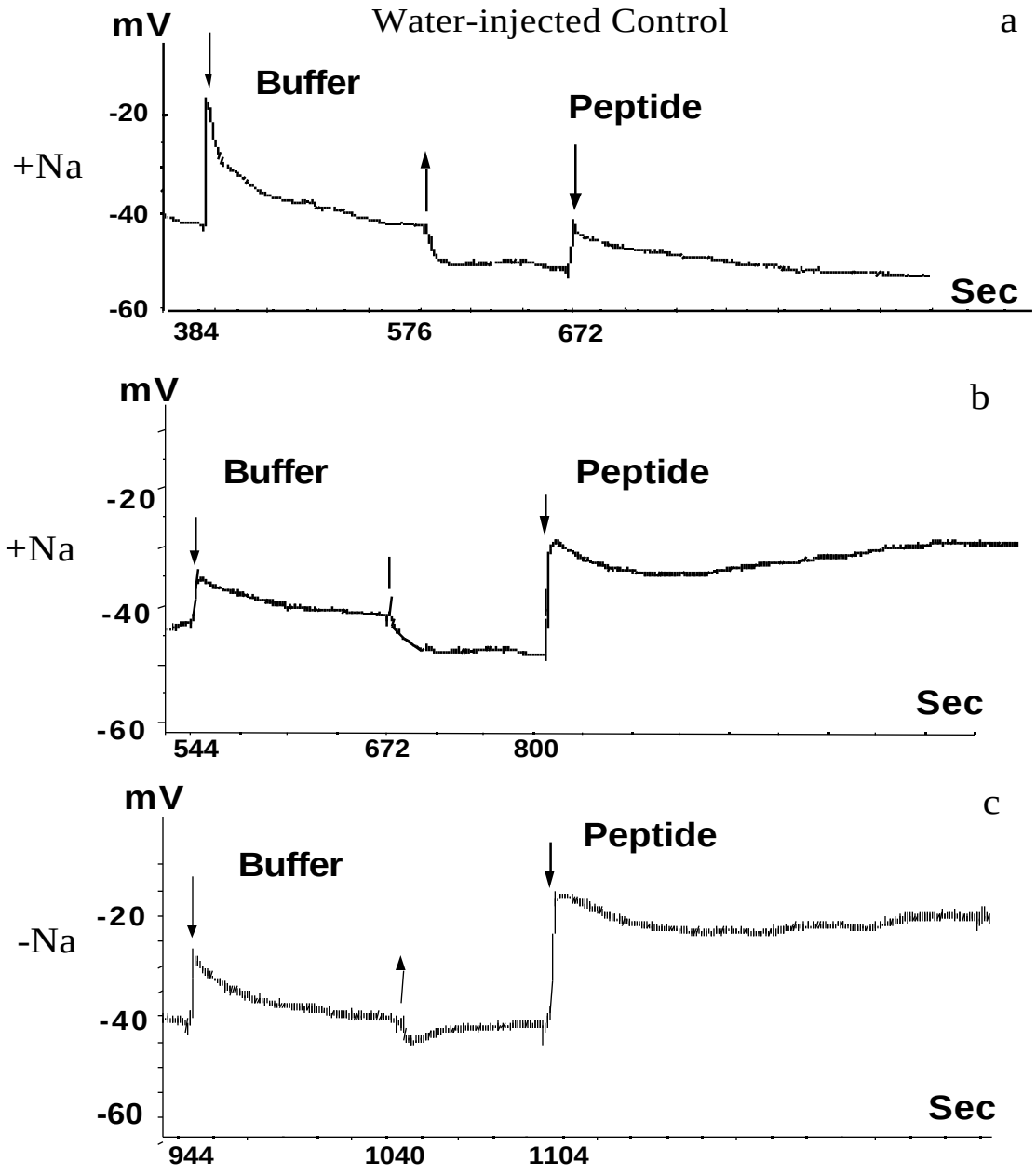

Figure 3.3. Na^+ dependence of responses to various peptides at pH 5.5 in *Xenopus* oocytes 5 to 7 d after injection with sheep omasal epithelial poly(A)$^+$ RNA or water. Examples of membrane potential changes in oocytes injected with water(a) or poly(A)$^+$ RNA(b, c) are shown. +Na and -Na indicate the recorded responses in Na^+-containing and Na^+-free solutions, respectively. For all figures, downward arrows(↓) indicate time of treatment addition, upward arrows(↑) indicate time of buffer removal.

Chapter IV

EPILOGUE

The results from the present research has demonstrated that there are mRNA present in sheep omasal epithelial cells that encode for peptide transporters by expression of these proteins in *Xenopus laevis* oocytes, and that peptide transport activity in RNA-injected oocytes can be detected by electrophysiological techniques.

These results are complementary to a previous study that specific poly(A)$^+$RNA fractions induced an increased rate of glycyl-sarcosine (Gly-Sar) absorption in mRNA-injected oocytes compared to water-injected oocytes (Matthews et al., 1996). This implies that a transport protein(s) that is capable of dipeptide absorption exists in sheep omasal epithelium.

The present study also, for the first time, revealed that a number of di-, tri-, and tetrapeptides with different amino acid compositions can serve as substrates for the potential omasal peptide transporter(s). If this is true within the animal body, the practical importance of this finding is that supplementation of amino acids in the form of peptides may be suggested. The present study was designed to identify the substrate specificity of the potential omasal peptide transporter(s) using an electrophysiological technique. Due to the poor expression of 150 ng total poly(A)$^+$ RNA in oocytes obtained by this method (evoked currents <20 nA), compared with up to 2,000 nA in oocytes injected with 50 ng of cRNA in another study of human intestinal peptide

transporter (Fei et al.,1994; Mackenzie et al., 1996), the cloning of DNA encoding proteins that transport peptides is necessary and critical to the study of N transport mechanisms.

However, in the current experiments, only a single electrode was used to detect the membrane potential change of the oocytes. The disadvantage of this technique is that most of the information obtained by this method is qualitative. In order to investigate the mechanisms of peptide transport mediated by the potential omasal peptide transporter(s) and describe the biochemical and kinetic characterization of the transporter(s), a two-microelectrode voltage-clamp technique should be developed for future studies.

The use of expression system to show the presence of functional omasal peptide transporter mRNA(s) does not directly demonstrate the presence of the transporter(s) in the omasal tissue. In order to demonstrate the presence of transport protein(s) in the omasal tissue, immunohistochemistry techniques need to be employed.

In general, this research has shown the transport ability and substrate specificity of a potential omasal transporter(s). This knowledge has the practical importance that an alternative supplementation of N source for animals may be identified.

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VITA

YuanXiang Pan, son of Guangyan Pan and Deyin Luo, was born on October 20, 1967, in Lanzhou, Gansu, P. R. of China. He was graduated from Lanzhou University, with a Bachelor of Science degree in Cell Biology in July, 1990. He married Hong Chen in August, 1992. He came to Virginia Tech in August, 1994 and initiated his program of graduate study in Protein Nutrition.

He is a member of The American Society of Animal Science, The American Institute of Nutrition, and Gamma Sigma Delta, the Honor Society of Agriculture

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YuanXiang Pan